

Open Research Online

The Open University's repository of research publications and other research outputs

Apolipoprotein D synthesis progressively increases in frontal cortex during human lifespan

Journal Item

How to cite:

Navarro, Ana; Del Valle, Eva; Juarez, Amalia; Martinez, Eva; Ordoñez, Cristina; Astudillo, Aurora and Tolivia, Jorge (2010). Apolipoprotein D synthesis progressively increases in frontal cortex during human lifespan. AGE - The Journal of the American Aging Association, 32(1) pp. 85–96.

For guidance on citations see [FAQs](#).

© 2010 American Aging Association

Version: Accepted Manuscript

Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.1007/s11357-009-9117-0>

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

oro.open.ac.uk

TITLE: Apolipoprotein D synthesis progressively increases in frontal cortex during human lifespan

AUTHORS: Ana Navarro*, Eva del Valle[§], Amalia Juárez*, Eva Martínez*, Cristina Ordóñez*, Aurora Astudillo[#] and Jorge Tolivia*

From:

(*) Departamento de Morfología y Biología Celular, Facultad de Biología y Medicina, Universidad de Oviedo, Spain

([§]) Life Sciences Department. The Open University. Walton Hall. Milton Keynes. Buckinghamshire. MK7 6AA. United Kingdom

([#]) Servicio de Anatomía Patológica, Hospital Central de Asturias, Spain

Corresponding author at:

Dr. Jorge Tolivia. Departamento de Morfología y Biología Celular. Facultad de Biología y Medicina. Universidad de Oviedo. Julián Clavería s/n. Oviedo 33006. Spain. (Tel: 348-5103061, Fax: 348-5103618).

E-mail address: jtolivia@uniovi.es

ABSTRACT

Apolipoprotein D (apo D) is a lipocalin present in the nervous system that may be related to processes of reinnervation, regeneration and neuronal cell protection. In the other way, apo D expression has been correlated, in some brain regions, with normal aging and neurodegenerative diseases. To elucidate the regional and cellular expression of apo D in normal human brain during aging, we performed a detailed and extensive study in samples of post-mortem human cerebral cortices. To achieve this study, slot blot techniques, for protein and mRNA, as well as immunohistochemistry and hybridohistochemistry methods were used. A positive correlation for apo D expression with aging was found; furthermore, mRNA levels,

as well as the protein ones, were higher in the white than in the grey matter. Immunohistochemistry and non-isotopic HIS showed that apo D is synthesized in both neurons and glial cells. Apo D expression is notorious in oligodendrocytes but with aging the number of neurons that synthesize apo D is increased. Our results indicate that apo D could play a fundamental role in central nervous system aging and in the reduction of products derivated from lipid peroxidation. The increment in the expression of apo D with aging can be included in a global mechanism of cellular protection to prevent the deleterious effects caused by aging.

KEYWORDS: apolipoprotein D, aging, human, frontal cortex, in situ hybridization, immunohistochemistry

ACKNOWLEDGMENTS

This work was supported by MEC and FEDER (SAF2007-64076/) grant. CO is a predoctoral fellow from “Gobierno del Principado de Asturias”, Spain. EM is a predoctoral fellow from “Ministerio de Educación y Ciencia”, Spain.

DISCLOSURE STATEMENT

All authors of the present work disclose that there are no actual and potential conflicts of interest.

The study achieved in the present work has approved by the Ethical Committee of Clinical Investigation of Asturias (Spain).

1. Introduction

Human apolipoprotein D (apo D), first isolated by McConathy and Alaupovic (1973; 1976) in plasma high-density lipoproteins (HDL), was classically considered a member of the apolipoproteins. However, further studies revealed that apo D belongs to 2-microglobulin super family or lipocalin family (Drayna et al. 1987), a group of small extra-cellular proteins involved in the transport of specific hydrophobic ligands (Flower 1996). This lipocalin is associated with LCAT in plasma, probably stabilizing the activity of the enzyme and helping in the efflux of cholesterol and its esters (Steyrer and Kostner 1988). Therefore, it has been hypothesized that the function of this protein could be cholesterol transport from peripheral tissues to liver for catabolism (Drayna et al. 1986). It has also been showed that apo D binds several hydrophobic ligands, including cholesterol, progesterone, pregnenolone, arachidonic acid, bilirubin and pheromones (Goessling and Zucker 2000; Morais-Cabral et al. 1995; Pearlman et al. 1973; Rassart et al. 2000; Zeng et al. 1996). In addition, apo D is expressed in a wide variety of tissues in mammals, like pancreas, kidney, placenta, adrenal gland, spleen and brain (Boyles et al. 1990b; Provost et al. 1991; Seguin et al. 1995; Smith et al 1990). The interstitial and connective fibroblasts near of blood vessels are the main cell type that accumulates apo D. A different local role for apo D depending on the tissue and the associated ligand has been proposed (Provost et al. 1991).

In the peripheral nervous system (PNS), apo D is synthesized and accumulated, as well as other apolipoproteins, by fibroblasts during regeneration following a lesion or axotomy (Boyles et al. 1990a; Del Signore et al. 2006; Spreyer et al. 1990) where it is thought to be implicated in the distribution of lipids during regeneration processes. Within central nervous system (CNS) apo D expression has been showed in pia matter cells, perivascular cells, pericytes, astrocytes, oligodendrocytes, and some scattered neurons (Navarro et al. 1998; Provost et al. 1991; Seguin et al. 1995; Smith et al. 1990). A difference in staining patterns

between glia and neurons has been observed in human brain regions (Navarro et al. 1998). Northern blot analysis of total mRNA extract from grey and white matters of human and rabbit brains showed that the white matter is the main site of apo D gene expression, which proves the hypothesis of apo D synthesis in fibrous astrocytes and oligodendrocytes (Provost et al. 1991). Furthermore, some studies have reported apo D mRNA in neuroglia and perivascular cells by *in situ* hybridization (Provost et al. 1991; Sanchez et al. 2002; Smith et al. 1990). It has been shown, *in vivo*, that astrocytes and fibroblast synthesize and constitutively secrete apo D under some particular conditions (Do Carmo et al. 2002; Patel et al. 1995). These authors suggest that apo D has a physiological role in cholesterol metabolism in nervous system. Neuronal synthesis of apo D has been reported as a suspicion but never has been confirmed (Provost et al. 1991; Smith et al. 1990).

A specific induction of apo D expression is observed during the repair following brain experimental injury in animals (Franz et al. 1999; Montpied et al. 1999; Ong et al. 1997; Rickhag et al. 2008; Terrisse et al. 1999; Trieu et al. 2000). The increase in apo D was mainly observed in astrocytes and neurons and recently in oligodendrocytes in injured regions (Rickhag et al. 2008). However, it is yet unknown whether these observations are due to an increased biosynthesis and /or uptake of apo D by these cells. The studies suggest that apo D could be a good marker of degeneration, being its expression induced as soon as any type of injury occurs.

In humans increased levels of apo D in brain have been demonstrated in several neuropathologies, such as Alzheimer disease (AD), scrapie, schizophrenia, etc (Dandoy-Dron et al. 1998; Terrisse et al. 1998; Thomas et al. 2001) as well as in normal aging (Belloir et al. 2001; Kalman et al. 2000). In AD, increased levels of apo D have been reported in CSF, hippocampus and cortex (Belloir et al. 2001; Kalman et al. 2000; Terrisse et al. 1998). Furthermore, not only neurons and reactive astrocytes but also some characteristic hallmarks

of AD as senile plaques and amyloid vessel deposits show apo D immunoreactivity (Desai et al. 2005; Del Valle et al. 2003; Navarro et al. 2001, 2003). In the same way, increased apo D expression has been demonstrated in pre-frontal cortex and caudate nucleus of subjects suffering from schizophrenia and bipolar disorders (Thomas et al. 2001). These findings suggest the hypothesis that apo D is a marker for brain regions that undergo some types of neuropathology (Thomas et al. 2001). Moreover, it could be part of a defence system acting in situations of unbalanced oxidative stress, which has been suggested, as an underlying mechanism in some neurodegenerative diseases (Muffat et al. 2008; Ordoñez et al. 2006; Sanchez et al. 2006). Like occurs in PNS, the increased apo D expression in CNS in these conditions allows to speculate a role in repair or remodelling processes secondaries to neurodegeneration (Boyles et al. 1990 a). Apo D may serve as a lipid carrier to and from cells and/or it may be a scavenger to deleterious molecules or free radicals like lipid peroxidation products (Ganforina et al. 2008).

Although recent studies reported that apo D expression is up regulated in aging and AD (Belloir et al. 2001; Kalman et al. 2000; Kang et al. 2003; Lee et al. 2000), detailed and extensive study in the course of adult human life has not been previously achieved. In this study, we have measured the levels of apo D protein and mRNA along human adult life in the grey as well as in the white matter of prefrontal cortex. In addition, protein and mRNA localization and distribution is described by hybrido - and immunohistochemistry methods.

2. Methods

2.1 Subjects

Human tissues were provided by the Department of Pathologic Anatomy of the Central Hospital of Asturias. Twenty-six cases (between 32 and 88 years) with not known neurological, psychiatric, or neuropathological disorders were used in this study (Mirra et al., 1991). Post-mortem intervals (PMI) ranged between 2 and 6 hours. Tissue blocks were obtained from human frontal cortex (Brodmann's area 9). The cerebral cortex was separated by dissection in white and grey matter. Three types of samples were obtained from each case, ones were frozen at -80°C for RNA or protein extraction and others were fixed in paraformaldehyde in 0.1 phosphate buffer (pH 7.4) for neuropathological diagnosis and immuno- or in situ hybrido-histochemical studies.

The present study was conducted according to the Declaration of Helsinki and has been approved by the local ethics committee.

2.2 Western blot analysis

Samples of grey and white matter from BA9 and samples from human breast cyst fluid (as a positive apo D control) were electrophoresed on a 10% SDS-PAGE in a Bio-Rad Miniprotean III electrophoresis unit. Subsequently, proteins were electrotransferred to a nitrocellulose membrane (Hybond ECL, GE healthcare) at 50V for 60 min. For avoiding non-specific reaction, membranes were blocked with TNT supplemented with 1% bovine serum albumin (BSA) for two hours at room temperature and then incubated with primary antibody against human apo D [1:10,000 dilution; antibody was a gift from Dr. Carlos López Otín, Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo (see Díez-Itza et al. 1994; López-Boado et al. 1994; Navarro et al. 1998, 2003, 2008; Ordóñez et al, 2006)] overnight at 4°C. After three washes of 10 min each in TNT membranes were incubated for 1

hour at room temperature with anti rabbit IgG HRP secondary antibody (Chemicon, 1:20,000 dilution). Membranes were washed again as we described above and proteins were detected by chemiluminescence using ECL reagent (GE healthcare) according to manufacture's instructions on a Kodak X-Omat film.

2.3 Protein slot-blotting.

Apo D levels were quantified using a slot blot technique. Samples from human breast cyst fluid were also included as a positive control of apo D and ExtrAvidin-Alkaline Phosphatase (Sigma, E26366) as an internal control. Briefly, 2, 1, 0.5, 0.25, 0.12, 0.06 µg of total protein were slot blotted under vacuum in a Bio-Dot (Biorad) apparatus. Nitrocellulose membranes were then immunostained for apo D protein according to the following protocol: non-specific binding was blocked by incubation with 1% BSA. Then they were incubated for 1 hour at room temperature with the antibody against human apo D (1:10,000 dilution). After 3 washes of 15 min each in PBS, membranes were incubated for 30 min at room temperature with biotinylated monoclonal anti-rabbit IgG (Sigma, B-5283) diluted 1:10,000, washed again as before and then incubated with ExtrAvidin-Alkaline Phosphatase (Sigma, E26366) diluted 1:10,000. After three washes of 15 min each in PBS enzyme activity was shown by incubation with Sigma Fast BCIP/NBT (Sigma, B5655) solution (2 hours at room temperature).

2.4 mRNA slot-blotting

Total RNA were extracted from dissected brain regions by homogenized by guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) and treated with RNase-free DNase I (GE Healthcare) in order to remove genomic DNA. ExtrAvidin-Alkaline Phosphatase was also included as an internal control. Briefly, 2, 1, 0.5, 0.25, 0.12, 0.06 µg of total RNA were denatured in 150µl of 20x SSC for 5 min at 70°C and

1 slot blotted under vacuum in a Bio-Dot (Biorad) apparatus. Nylon membranes (Hybond-N+
2 GE Healthcare,) were then prehybridized at 56°C for 2 hours in Rapid-hyb buffer (GE
3 Healthcare). Hybridization in the same buffer was then performed over night using 5 µl of
4 digoxigenin-labeled RNA probe for human apo D (obtained from Dr. Carlos López-Otín,
5 Dpto. Bioquímica y Biología Molecular, Universidad de Oviedo; see [Del Valle et al. 2003;
6 Díez-Itza et al. 1994; López-Boado et al. 1994]). After several washes in TNT (3x15 min),
7 hybridized probe was detected with an AP-coupled anti-DIG antibody (Roche 1093274)
8 diluted 1:10,000 for 30 min at room temperature. For signal amplification the membranes
9 were rinsed with TNT (3x10 min) and treated with biotinylated universal antibody (BA-1300,
10 Vector Laboratories, Burlingame, CA) diluted 1:10,000, and ExtrAvidin-Alkaline
11 Phosphatase (Sigma, E26366) diluted 1:10,000, both for 30 min at room temperature. Enzyme
12 activity was shown by incubation with Sigma Fast BCIP/NBT (Sigma, B5655) solution (2
13 hours at room temperature).

14 The blots of both, protein and mRNA of apo D, were dried and bands were quantified
15 as a relative optical densities (ROD) (arbitrary units) using a digital scanner (Nikon AX-110,
16 Nikon) and NIH Image1.57 free software (Scion Corp).

17 2.5 Hybridohistochemistry and immunohistochemistry

18 After fixation, blocks were washed in distilled water, dehydrated, cleared in butyl
19 acetate and embedded in paraffin. Sections were cut at 10 µm in thickness and mounted on
20 "SuperFrost Plus" (Mentzel-Glasse) slides, dried at 36°C for 24 hours, deparaffined in xylene,
21 and rehydrated by successive alcohols for 2 min each. Endogenous peroxidase was blocked by
22 subsequent incubation in 3% hydrogen peroxide solution.

23 *In situ* hybridization was performed on sections dried at room temperature for 5 min,
24 digested with 2 µg/ml of proteinase K in 0.001M PBS at 37°C for 5 min, and rinsed in 0.001M

PBS. Twenty μ l of ISH solution (Sigma Hybridization Solution, H-7782) were applied on each section for 10 min. Twenty μ l of digoxigenin-labeled RNA probe for human apo D were applied on each of the sections that subsequently were incubated in a moist chamber for 16 hours at 55°C. The hybridized probe was visualized by two methods of detection. (1) The slides were rinsed in PBS and the hybridized probe was detected with an alkaline phosphatase-coupled anti-DIG antibody (Roche 1093274) diluted 1:1,000 for 60 min at room temperature. Then, slides were rinsed with PBS and incubated in Sigma Fast BCIP/NBT (Sigma B5655) solution (14 hours at room temperature). (2) The slides were rinsed in PBS and the hybridized probe was detected with an HRP-coupled anti-DIG antibody (Roche 1207733) diluted 1:500 for 60 min at room temperature. For the signal amplification, the sections were rinsed with PBS and treated with "Vectastain Universal Quick kit" (PK-8800, Vector Laboratories). Peroxidase activity was shown by incubation with sigma Fast DAB (Sigma D4168) at room temperature for 30 min.

For immunohistochemistry, sections were treated with triton X (0.1%, five min), washed in distilled water, treated with H₂O₂ (3%, five min), washed in distilled water and treated with PBS. Non-specific binding was blocked by incubation with BSA (30 min). Incubation with a specific antibody against human apo D (1:2,000 dilution) was carried out overnight at 4°C. The immunoreactivity was detected using the Extravidin-biotin-peroxidase staining kit (Sigma Extra-3) and peroxidase activity was shown as it was described above for hybridohistochemistry.

Finally, the sections visualized with DAB were counterstained with a modification of formaldehyde-thionin method (Tolivia et al. 1994), dehydrated, cleared in eucalyptol and mounted with Eukitt. The sections revealed with NBT were mounted in aqueous mounting medium. The usual specificity control tests for both, hybridohistochemistry and immunohistochemistry, were carried out.

2.6 Statistical analyses

The data were subjected to a statistic study using SPSS 15.0 for Windows. The test of Kolmogorov-Smirnov with the correction of Lilliefors was used to evaluate the fit of the data to a normal distribution. In view of no homogeneity of variance, we performed the Mann-Whitney test to compare the variables (apo D protein and apo D mRNA) between white and grey matter. P values <0.05 were considered as statistically significant. Finally, a correlation analysis and a lineal regression were done to detect the association between age and protein and mRNA apo D levels, respectively.

3. Results

3.1. Western blot for apo D

In the present work, we have measured apo D gene expression and its cellular and tissue localization in human frontal cerebral cortex during adult lifespan and aging, in white and grey matter samples. As it is shown in Fig. 1, western blot technique clearly demonstrates a band between 29 and 31 KDa, corresponding to apo D, in all cerebral extracts analyzed. Our results (Fig. 1 A, B) show that apo D is expressed in all samples, white and grey matter, young and elders.

In the grey matter, a clear tendency to an apo D increment related with age can be observed (Fig. 1 A). In spite of this bias is also present in the white matter samples it shows more oscillations than in grey matter (Fig. 1 B). When samples of the same subject, are compared, the white matter shows the higher amount of apo D (Fig. 1 A, B).

3.2. Apo D protein levels

The levels of expression of apo D were measured in all frontal cortex samples to study direct effects of age. Slot blot technique showed similar results to that observed in the western blot (Fig. 2). The analysis of the densitometric values of the slots showed that apo D protein levels are increased with a gradual pattern during aging in both, grey and white matter. However, apo D is expressed in grey and white matter in different manner. The highest levels of protein were always seen in white matter samples (Fig. 3), just as we had appreciated in western and slot blot images. In addition, grey matter presented more individual oscillations than white matter (Fig. 3). A positive correlation was found in both white and grey matter between apo D level and age ($r = 0.860$; $p < 0.001$ and $r = 0.606$; $p < 0.01$, respectively). In all decades a statistically significative difference in apo D levels between white and grey mater is

observed, being the protein levels in the white matter higher in all the cases. Notwithstanding, the differences are less pronounced in the middle age decades.

3.3. Tissue distribution of apo D protein

The protein slot-blot results showed that apo D is present in white and grey matter in human frontal cortex in all ages under study, so we decided to examine the distribution and cellular localization of this protein using immunohistochemistry on representative sections of the same brains. In general, a clear tendency to the increment of apo D positive staining with age is observed (Fig. 4). In young subjects, apo D immunostaining in grey matter is scarce and mainly located in glial and meningeal cells as well as in some perivascular cells (Fig. 4 A), whereas in white matter is higher and oligodendrocytes are the main labelled cells (Fig. 4 F). During aging, the number of immunoreactive cells and the intensity of extracellular signal are increased in the white matter (Fig. 4 G). Immunoreactivity for apo D in the grey matter is found in a small number of astrocytes and oligodendrocytes down to the middle age (Fig. 4 A). However, in elders, an increase in the number of oligodendrocytes and astrocytes labelled for apo D can be clearly observed and the intensity of signal is higher than in the young ones (Fig. 4 B).

There are not apo D reactive neurons in the youngest samples but in some individual cases a weakly signal for apo D can be detected (Fig. 4 C) with aging some neurons, principally pyramidal cells, appear labelled and over the sixth decade a great number of neuronal cells are immunopositives for apo D (Fig. 4 B, E). Positive neurons are not usually linked to any aging or pathological related features and always show microscopical characteristics of a normal neuron (vesicular nucleus, large nucleolus and the presence of granular or striated Nissl substance in the cytoplasm) (Fig. 4 C, E). Apo D immunoreactivity was also found in blood vessels, mainly in pial and subpial layer (Fig. 4 A) and occasionally

1 in cortical and sub-cortical layer. Number and signal intensity of pial and subpial blood
2 vessels are increased during aging while cortical and sub-cortical vessels did not. Apo D
3
4 signal was uniform around small blood vessels and was discontinue in big vessels. It could be
5
6 observed inside perivascular cells (pericytes and fibroblasts) but is undetectable in endothelial
7
8 cells (Fig. 4 D).
9
10

11 12 13 14 15 3.4. mRNA slot-blot 16

17 Gene expression of apo D in the frontal cortex in all subjects was measured using slot
18 blot technique (Fig. 2). When the densitometric values of the slots were analysed, our results
19
20 showed a gradual and significant increase of apo D mRNA during aging in both, grey and
21
22 white matter, but in different fashion. The highest levels of mRNA were always seen in white
23
24 matter samples as we had also observed for the protein (Fig. 5). In addition, white matter
25
26 levels of mRNA presented less individual oscillations than grey matter ones. Data
27
28 demonstrated that the increase of apo D mRNA with age is statistically significative in grey
29
30 ($r= 0.555$, $p<0.05$) as well as in white matter ($r=0.518$, $p<0.01$). Comparative studies of
31
32 expression for apo D showed statistical differences between grey and white matter in all
33
34 decades, being levels always higher in white matter.
35
36
37
38
39
40
41
42
43
44

45 3.5. Tissue distribution of the apo D mRNA 46

47 *In situ* non-radioactive hybridization was subsequently performed to determine
48
49 localization of apo D mRNA in human frontal cortex. In younger subjects, the signal in this
50
51 cortical region is restricted to the white matter while grey matter presents faint signal. In the
52
53 white matter, the number of labelling glial cells is increased with age, showing a stronger
54
55 signal in elders (Fig. 6 A). These cells are mainly oligodendrocytes, recognized for their
56
57 morphological characteristics (Fig. 6 B). In the grey matter, we also observed an increase of
58
59
60
61
62
63
64
65

1 labelled cells with aging. These cells were identified as neurons and astrocytes (Fig.6 C-E) but
2 expression signal was also detected in microglia and pericytes (Fig. 6 F). Neurons showing
3 mRNA for apo D are principally pyramidal cells (Fig. 6 C, D) but granular neurons could be
4 also detected (Fig. 6 E). Positive neurons always show microscopical characteristics of a
5 normal neuron (vesicular nucleus, large nucleolus) (Fig. 6 D, E). The deposits of chromogen
6 (Fig. 6 D) mask the presence of granular or striated Nissl substance in the cytoplasm. The
7 numbers of hybridized neurons increased with aging as we also have seen with
8 immunohistochemistry.
9
10
11
12
13
14
15
16
17
18
19
20
21

22 *3.6. Protein and mRNA relation*

23
24 To complete the study we achieved a statistical analysis to determine the correlation
25 between mRNA and protein levels in white and grey matter (Fig. 7 A, B). The result of
26 protein closely correspond with mRNA immunoblots in white ($r= 0.454$; $p= 0.05$) and grey
27 matter ($r= 0.522$; $p= 0.01$).
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

4. Discussion

Several studies relate increments in the expression of apo D in brain regions affected by neurological diseases. This relation has led to the hypothesis that apo D could be a marker for brain neuropathology. Increases in apo D expression selectively within CNS injured regions suggest a focal compensatory response of apo D (Franz et al. 1999; Terrisse et al. 1999; Thomas et al. 2001). However, few data have been reported about the amounts of apo D in brain of normal subjects. The use of human tissue as study material has very important associated difficulties derived to each particular case (e.g., cause of death, life stiles, differences of medication), however it is required to obtain direct information about how apo D behaves during lifetime. The value of our work lies in to take samples in multiple time points during human development and normal aging and to perform quantitative and qualitative analyses of apo D protein and mRNA in all of them. Tissue blocks were divided in grey and white matter that allowed us to obtain separate results of both structures.

Protein measurement by immunoblotting showed that in spite of quantitative differences were found between levels of apo D in the white and grey matter in frontal cortex both increase with age. Immunohistochemical analyses showed the expected cellular localization in oligodendrocytes, astrocytes and neurons, and an increment in the number of positive cells with aging. According to Kalman et al. (2000), these results suggest that apo D protein is involved in normal aging process. Senile plaques and vascular amyloid were also stained although neurofibrillar tangles were not. Apo D immunoreactivity was found mainly in the nonconophilic parts of vessels and plaques as was previously published (Navarro et al. 2003). The presence of apo D in some pathological markers of AD could be an indirect consequence of the aging process more than a contribution of apo D to the pathophysiology of the disease.

Studies carried out by Northern blot and isotopic ISH in prefrontal cortex of elders with and without Alzheimer disease (AD) showed apo D labelling for all cases and regions under study always linked to white matter and its glial cells (Belloir et al. 2001; Provost et al. 1991). Moreover, increments of mRNA occur only in those subjects with the pathology (Belloir et al. 2001). Our study shows an increase of apoD mRNA during aging, this increment is bigger in grey matter where we have seen that the percentage of apo D labelled astrocytes and neurons grow. The anisotopic signal found in neurons show, for the first time, that apo D could be expressed and synthesized by those nervous cells. That expression was supposed, but not clearly confirmed, by other authors (Smith et al. 1990; Belloir et al. 2001), in monkey and humans using isotopic hybridization methods. Our results suggest that neurons of some encephalic regions not only can obtain apo D from the surrounding glia but also express this protein as response to changes in the cellular homeostasis during aging. However some neuronal nuclei or individual neurons cannot express apo D, as was previously published by our group (Navarro et al. 2008; Ordóñez et al. 2006), and these nervous cells show a great vulnerability to the stressors during aging and under pathologic situations.

In our study, western and slot blots showed that the amount of apo D protein and mRNA measured was always higher in the white that in the grey matter. In addition, an increment in the amount of protein and mRNA is observed during lifespan. Using microarray technology Loerch et al. (2008) have shown a strong up-regulation of the apo D gene in aging, not only in humans, but also in rhesus monkey and mouse, being the most phylogenetically conserved in the three species among the so-called “aging genes”. The presence and expression of apo D in oligodendrocytes and the increased signal observed in this cellular type in the elderly subjects suggest a fundamental role for these cells in the normal aging process.

In conclusion, the present work describes a progressive increment in both apo D protein and mRNA levels in frontal cortex (Brodmann’s area 9) of normal subjects during

1 the normal aging process. Increments of apo D and/or its mRNA have been reported in the
2 CSF, hippocampus, frontal and temporal cortex and entorhinal cortex of AD subjects, whereas
3
4 in other areas, as parietal cortex and cerebellum, quantitative differences were not observed
5
6
7 (Belloir et al. 2001; Kalman et al. 2000; Navarro et al. 1998; Thomas et al. 2003). Thus, some
8
9 brain regions habitually present apo D whereas others do not. The region-specific distribution
10
11 for apo D expression was introduced by previous studies of our group (Navarro et al. 1998)
12
13 and later reported by Thomas et al. (2001, 2003). These authors found that the increase of apo
14
15 D level was associated in some brain regions with the pathophysiology of AD, schizophrenia
16
17 and bipolar disorder. However, these data are controversial because no statistical differences
18
19 where found by immunoblotting when compared AD with age-matched control subjects
20
21
22 (Kalman et al. 2000). Recent studies show that those differences in expression of apo D exist
23
24 among neuronal nuclei located in the same region (Ordóñez et al. 2006) or among neurons of
25
26 the same area (Navarro et al. 2008). Based in these observations, to keep an apo D profile of
27
28 brain regions is important to interpret experimental and pathological data.
29
30
31
32
33
34

35 A great number of studies show that the normal aging, as well as some
36
37 neurodegenerative disorders, are related with the oxidative metabolism of different molecules.
38
39 The new molecules formed can damage, directly or indirectly, the cellular homeostasis and as
40
41 a consequence, the cell dies. In the last years, it has been proved that apo D is a protein
42
43 directly related with the oxidative stress. Studies achieved in *Drosophila* show that its absence
44
45 reduces lifespan and stress resistance (Sanchez et al. 2006) and, on the contrary, the over-
46
47 expression of apo D extends lifespan and increases stress resistance (Muffat et al. 2008).
48
49 These results are in concordance with other studies that show that its expression or over-
50
51 expression has a neuroprotective effect and protect nervous cells from some damages
52
53 (Navarro et al. 2008; Ordóñez et al. 2006; Sanchez et al. 2006; Walker et al. 2006). These
54
55 authors say that apo D could play an important role in the reduction of products derivatives of
56
57
58
59
60
61
62
63
64
65

the lipid peroxidation. The over-expression of apo D with aging, in the cerebral cortex, can be included in a global mechanism of cellular protection used by the nervous cells to prevent the deleterious effects of lipid peroxidation caused by ageing.

5. Reference list

- Belloir B, Kovari E, Surini-Demiri M, Savioz, A (2001) Altered apolipoprotein D expression in the brain of patients with Alzheimer's disease. *J Neurosci Res* 64: 61-69
- Boyles JK, Notterpek LM, Anderson LJ (1990a) Accumulation of apolipoproteins in the regenerating and remyelinating mammalian peripheral nerve. Identification of apolipoprotein D, apolipoprotein A-IV, apolipoprotein E and apolipoprotein A-I. *J Biol Chem* 265: 17805-17815
- Boyles JK, Notterpek LM, Wardell MR, Rall SC Jr (1990b) Identification, characterization, and tissue distribution of apolipoprotein D in the rat. *J Lipid Res* 31: 2243-2256
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159
- Dandoy-Dron F, Guillo F, Benboudjema L, Deslys JP, Lasmézas C, Dormont D, Tovey MG, Dron M (1998) Gene expression in scrapie. Cloning of a new scrapie-responsive gene and the identification of increased levels of seven other mRNA transcripts. *J Biol Chem* 273: 7691-7697
- Del Signore A, De Sanctis V, Di Mauro E, Negri R, Perrone-Capano C, Paggi P (2006) Gene expression pathways induced by axotomy and decentralization of rat superior cervical ganglion neurons. *Eur J Neurosci* 23: 65-74
- Del Valle E, Navarro A, Astudillo A, Tolivia J (2003) Apolipoprotein D expression in human brain astrocytes. *J Histochem Cytochem* 51: 1285-1290
- Desai PP, Ikonomic MD, Abrahamson EE, Hamilton RL, Isanski BA, Hope CE, Klunk WE, DeKosky ST, Kamboh MI (2005) Apolipoprotein D is a component of compact but not diffuse amyloid-beta plaques in Alzheimer's disease temporal cortex. *Neurobiol Dis* 20: 574-82

Díez-Itza I, Vizoso F, Merino AM, Sánchez LM, Tolivia J, Fernández J, Ruibal A, López-Otín C (1994) Expression and prognostic significance of apolipoprotein D in breast cancer. *Am J Pathol* 144: 310-320

Do Carmo S, Séguin D, Milne R, Rassart E (2002) Modulation of apolipoprotein D and apolipoprotein E mRNA expression by growth arrest and identification of key elements in the promoter. *J Biol Chem* 277: 5514-5523

Drayna D, Fielding C, McLean J, Baer B, Castro G, Chen E, Comstock L, Henzel W, Kohr W, Rhee L, Wion K, Lawn K (1986) Cloning and expression of human apolipoprotein D cDNA. *J Biol Chem* 261: 16535-16539

Drayna DT, McLean JW, Wion KL, Trent JM, Drabkin HA, Lawn RM (1987) Human apolipoprotein D gene: gene sequence, chromosome localization, and homology to the alpha 2micro-globulin superfamily. *DNA* 6: 199-204

Franz G, Reindl M, Patel SC, Beer I, Unterrichter I, Berger T, Schmutzhard E, Poewe W, Kampfl A (1999) Increased expression of apolipoprotein D following experimental traumatic brain injury. *J Neurochem* 73: 1615-1625

Flower DR (1996) The lipocalin protein family: structure and function. *Biochem. J.* 318: 1-14.

Ganfornina MD, Do Carmo S, Lora JM, Torres-Schumann S, Vogel M, Allhorn M, González C, Bastiani MJ, Rassart E, Sanchez D (2008) Apolipoprotein D is involved in the mechanisms regulating protection from oxidative stress. *Aging Cell* 7: 506-515

Goessling W, Zucker SD (2000) Role of apolipoprotein D in the transport of bilirubin in plasma. *Am. J. Physiol. Gastrointest. Liver Physiol* 279: G356-65

Kalman J, McConathy W, Araoz C, Kasa P, Lacko AG (2000) Apolipoprotein D in the aging brain and in Alzheimer's dementia. *Neurol Res* 22: 330-336

Kang MK, Kameta A, Shin KH, Baluda MA, Kim HR, Park NH (2003) Senescence-associated genes in normal human oral keratinocytes. *Exp Cell Res* 287: 272-281

1 Lee CK, Weindruch R, Prolla TA (2000) Gene-expression profile of the ageing brain in mice.
2 Nat Genet 25: 294-297
3
4 Loerch PM, Lu T, Dakin KA, Vann JM, Isaacs A, Geula C, Wang J, Pan Y, Gabuzda DH, Li
5 C, Prolla TA, Yankner BA (2008) Evolution of the aging brain transcriptome and synaptic
6 regulation. PLoS ONE 3: e3329
7
8 López-Boado YS, Tolivia J, López-Otín C (1994) Apolipoprotein gene induction by retinoic
9 acid is concomitant with growth arrest and cell differentiation in human breast cancer cells. J
10 Biol Chem 269: 26871-26878
11
12 McConathy WJ, Alaupovic P (1973) Isolation and partial characterization of apolipoprotein
13 D: a new protein moiety of the human plasma lipoprotein system. FEBS Lett 37: 178-182
14
15 McConathy WJ, Alaupovic P (1976) Studies on isolation and partial characterization of
16 apolipoprotein D and lipoprotein D of human plasma. Biochemistry 15: 515-520
17
18 Mirra SS, Heyman A, McKeel D (1991) The Consortium to Establish a Registry for
19 Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of
20 Alzheimer's disease. Neurology 41: 479-486
21
22 Montpied P, de Bock F, Lerner-Natoli M, Bockaert J, Rondouin G (1999) Hippocampal
23 alterations of apolipoprotein E and D mRNA levels in vivo and in vitro following kainate
24 excitotoxicity. Epilepsy Res 35:135-146
25
26 Morais-Cabral JH, Atkins GL, Sánchez LM, Lopez-Boado YS, Lopez-Otin C, Sawyer L
27 (1995) Arachidonic acid binds to apolipoprotein D: implications for the protein's function.
28 FEBS Lett 366: 53-56
29
30 Muffat J, Walker DW, Benzer S (2008) Human ApoD, an apolipoprotein up-regulated in
31 neurodegenerative diseases, extends lifespan and increases stress resistance in *Drosophila*.
32 Proc Natl Acad Sci U S A 105: 7088-7093
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55

Rickhag M, Deierborg T, Patel S, Ruscher K, Wieloch T (2008) Apolipoprotein D is elevated
 in oligodendrocytes in the peri-infarct region after experimental stroke: influence of enriched
 environment. *J Cereb Blood Flow Metab* 28: 551-562

Sánchez D, Ganfornina MD, Martínez S (2002) Expression pattern of the lipocalin
 apolipoprotein D during mouse embryogenesis. *Mech Dev* 110: 225-229

Sánchez D, López-Arias B, Torroja L, Canal I, Wang X, Bastiani MJ, Ganfornina MD (2006)
 Loss of glial lazaro, a homolog of apolipoprotein D, reduces lifespan and stress resistance in
Drosophila. *Curr Biol* 16: 680-686

Seguin D, Desforges M, Rassart E (1995) Molecular characterization and differential mRNA
 tissue distribution of mouse apolipoprotein D. *Mol Brain Res* 30: 242-250.

Smith KM, Lawn RM, Wilcox JN (1990) Cellular localization of apolipoprotein D and
 lecithin:cholesterol acyltransferase mRNA in rhesus monkey tissues by in situ hybridization. *J*
Lipid Res 31: 995-1004

Spreyer P, Schaal H, Kuhn G, Rothe T, Unterbeck A, Olek K, Muller HW (1990)
 Regeneration-associated high level expression of apolipoprotein D mRNA in endoneurial
 fibroblasts of peripheral nerve. *EMBO J* 9: 2479-2484

Steyrer E, Kostner GM (1988) Activation of lecithin-cholesterol acyltransferase by
 apolipoprotein D: comparison of proteoliposomes containing apolipoprotein D, A-I or C-I.
Biochim Biophys Acta 958: 484-491

Terrisse L, Poirier J, Bertrand P, Merched A, Siest G, Milne RW, Rassart E (1998) Increased
 levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. *J.*
Neurochem 71: 1643-1650

Terrisse L, Seguin D, Bertrand P, Poirier J, Milne RW, Rassart E (1999) Modulation of
 apolipoprotein D and apolipoprotein E mRNA expression in rat hippocampus after entorhinal
 cortex lesion. *Mol Brain Res* 70: 26-35

1 Thomas EA, Dean B, Pavey G, Sutcliffe JG (2001) Increased CNS levels of apolipoprotein D
 2 in schizophrenic and bipolar subjects: implications for the pathophysiology of psychiatric
 3 disorders. *Proc Natl Acad Sci U S A* 98: 4066-4071
 4
 5 Thomas EA, Laws SM, Sutcliffe JG, Harper C, Dean B, McClean C, Masters C,
 6 Lautenschlager N, Gandy SE, Martins RN (2003) Apolipoprotein D levels are elevated in
 7 prefrontal cortex of subjects with Alzheimer's disease: no relation to apolipoprotein E
 8 expression or genotype. *Biol Psychiatry* 54: 136-141
 9
 10 Tolivia J, Navarro A, Tolivia D (1994) Differential staining of nerve cells and fibres for
 11 sections of paraffin-embedded material in mammalian central nervous system. *Histochemistry*
 12 102: 101-104
 13
 14 Trieu VN, Uckun FM (2000) Apolipoprotein E and apolipoprotein D expression in a murine
 15 model of singlet oxygen-induced cerebral stroke. *Biochem Biophys Res Commun* 268:
 16 835-841
 17
 18 Walker DW, Muffat J, Rundel C, Benzer S (2006) Overexpression of a Drosophila homolog of
 19 apolipoprotein D leads to increased stress resistance and extended lifespan. *Curr Biol*.16: 674-
 20 679
 21
 22 Zeng C, Spielman AI, Vowels BR, Leyden JJ, Biemann K, Preti G (1996) A human axillary
 23 odorant is carried by apolipoprotein D. *Proc. Natl. Acad. Sci. USA* 93: 6626-6630
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65

Figures

Figure 1. - Western blot analysis in grey (A) and white (B) matter of apo D in cerebral cortex from six subjects of different age (82-37 years old). The line 7 was charged with breast cyst fluid.

Figure 2. - Slot blot analysis of apo D protein and for its mRNA from two subjects (32 and 72 years old). Samples from white and grey matter are included and the differences during aging and between nervous regions can be clearly observed.

Figure 3. - Levels of apo D, in white (WM) and grey matter (GM), in samples from 26 subjects between 32 and 88 years old. The histogram shows the densitometry analysis of slot blots. Data presented as means of relative optical densities (ROD) with standard error of the mean shown by vertical bars. The lineal correlation with age it is also shown ($r = 0.860$; $p < 0.001$ for the white matter and $r = 0.606$; $p < 0.01$ for the grey matter).

Figure 4. - Immunocytochemistry for apo D on grey (A-E) and white matter (F, G). A) Cerebral cortex of young subject, some glial cells and one vessel are marked. B) Cerebral cortex of old subject, a great number of neurons and glial cells are positives for apo D. C) One neuron in the grey matter of a young subject shows a few grains of apo D. D) Vessel in the cerebral cortex (fig. 4 A) showing positive signal in perivascular cells but not in endothelial cells. E) Positive neurons in cerebral cortex of an old subject, some glial cells can be also observed. F) White matter from a young subject, a general positive signal for apo D can be observed. G) White matter from an old subject, an intense signal for apo D principally located in the oligodendrocytes is showed.

Figure 5. - Levels of apo D mRNA, in white (WM) and grey matter (GM), in samples from 26 subjects between 32 and 88 years old. The histogram shows the densitometry analysis of slot blots. Data presented as means of ROD with standard error of the mean shown by vertical bars.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

The lineal correlation with age it is also shown in grey ($r= 0.555$; $p<0.05$ for the grey matter and $r=0.518$; $p<0.01$ for the white matter).

Figure 6. - Hybridocytochemistry for apo D mRNA on white (A, B) and grey matter (C-F). A) White matter from an old subject, an intense expression of apo D can be observed. B) White matter from an old subject, two oligodendrocytes expressing apo D are showed. C) Cerebral cortex of an old subject, a great number of pyramidal neurons expressing apo D can be observed. D) Pyramidal neuron expressing apo D in which the normal morphology of nucleus and nucleoli can be observed. E) Granular neurons in cerebral cortex of an old subject expressing apo D. F) Pericyte and astrocyte cells expressing apo D in an old subject.

Figure 7. - Correlation between mRNA and apo D protein levels during aging in white (A) ($r= 0.454$; $p= 0.05$) and grey matter (B) ($r= 0.522$; $p= 0.01$).

Figure 1

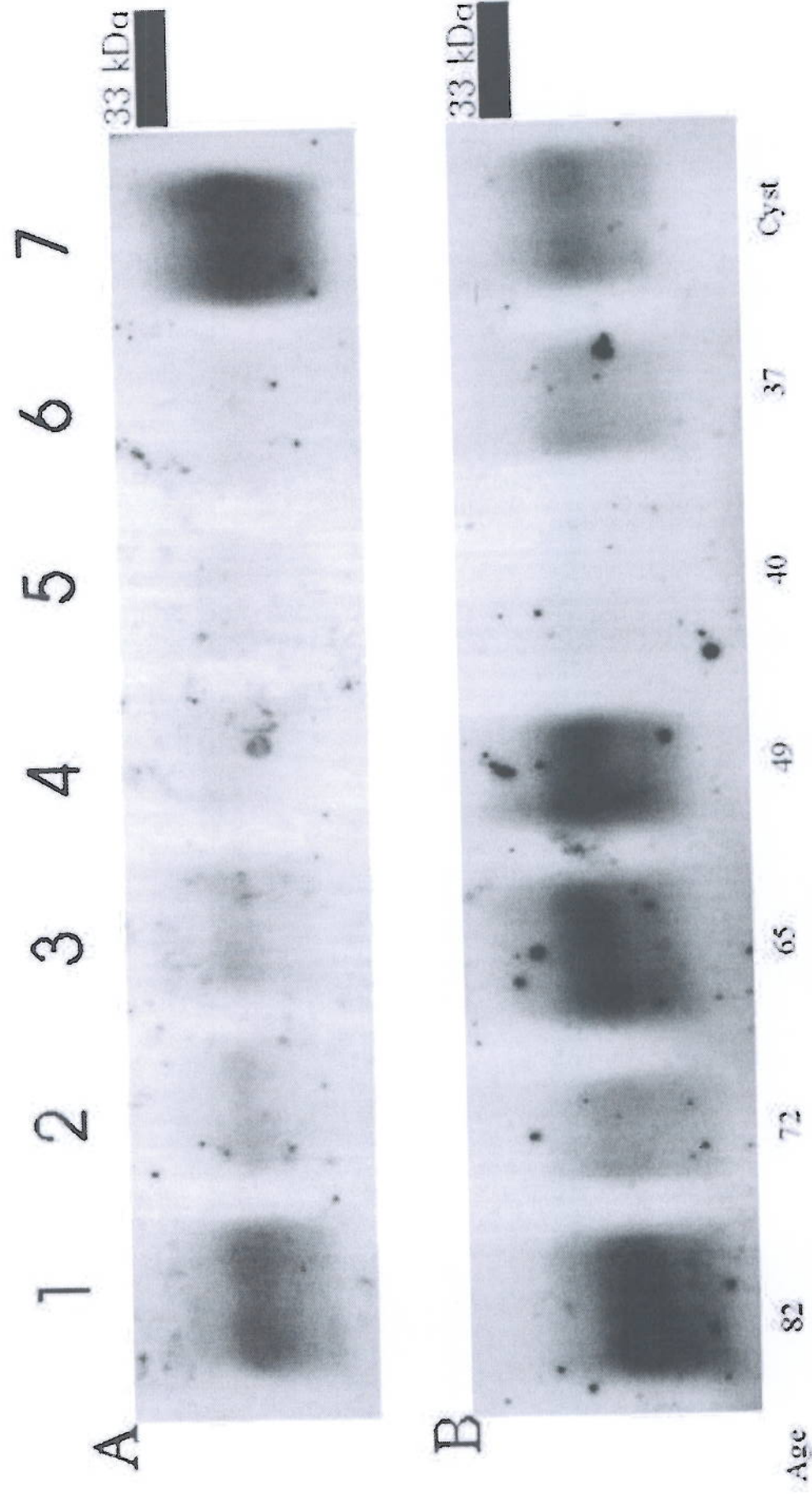


Figure 2

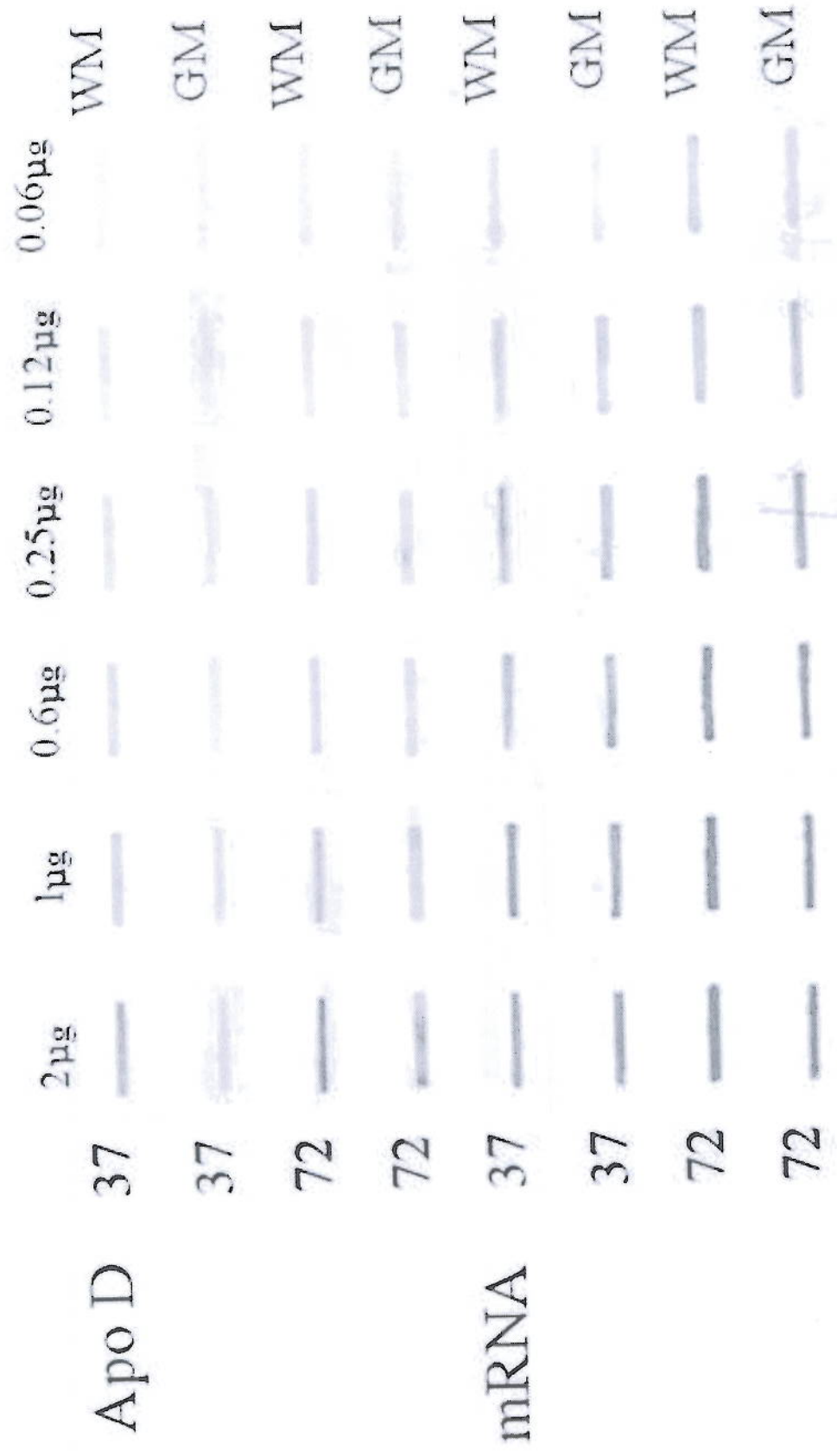


Figure 3

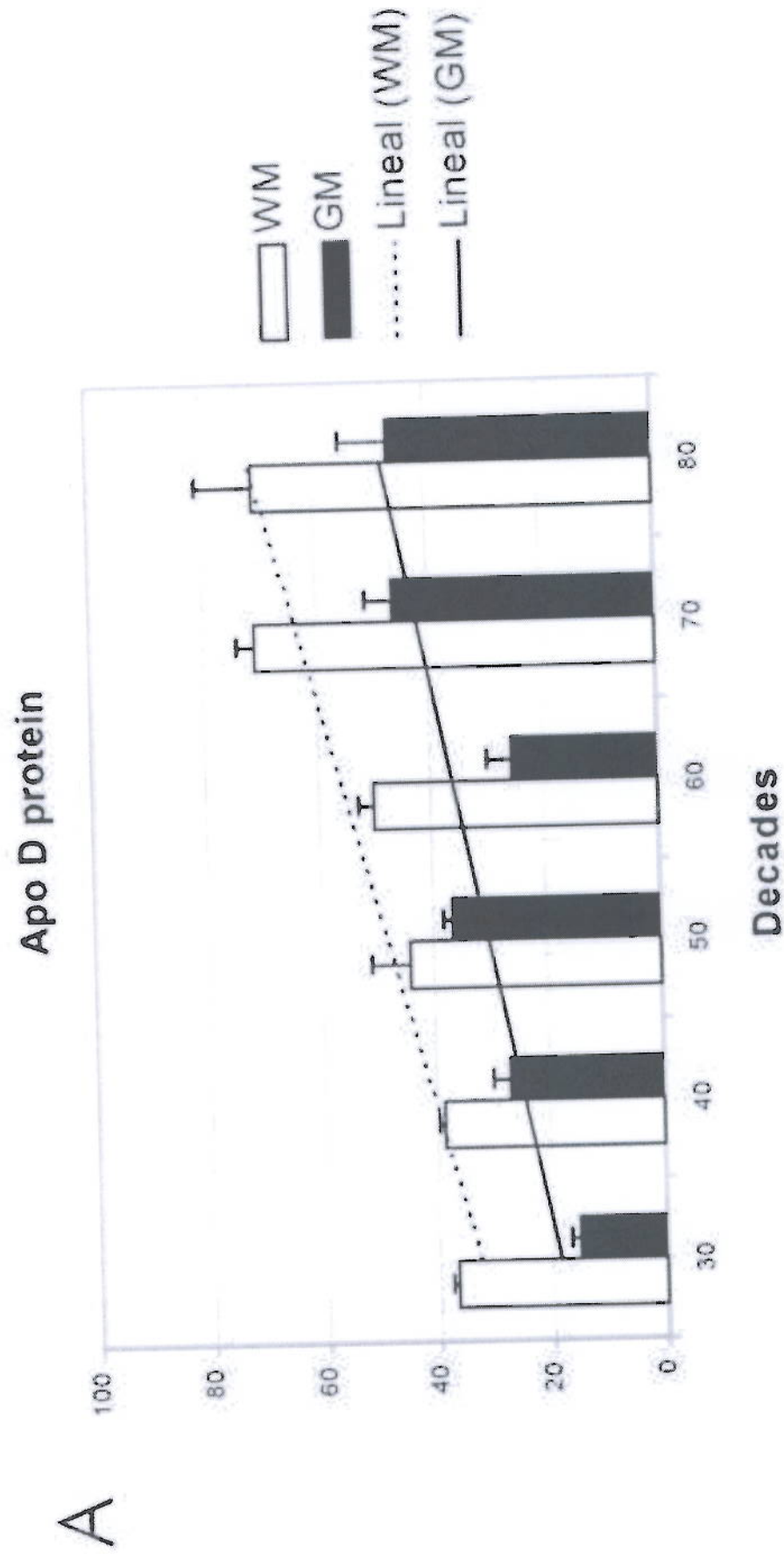


Figure 4

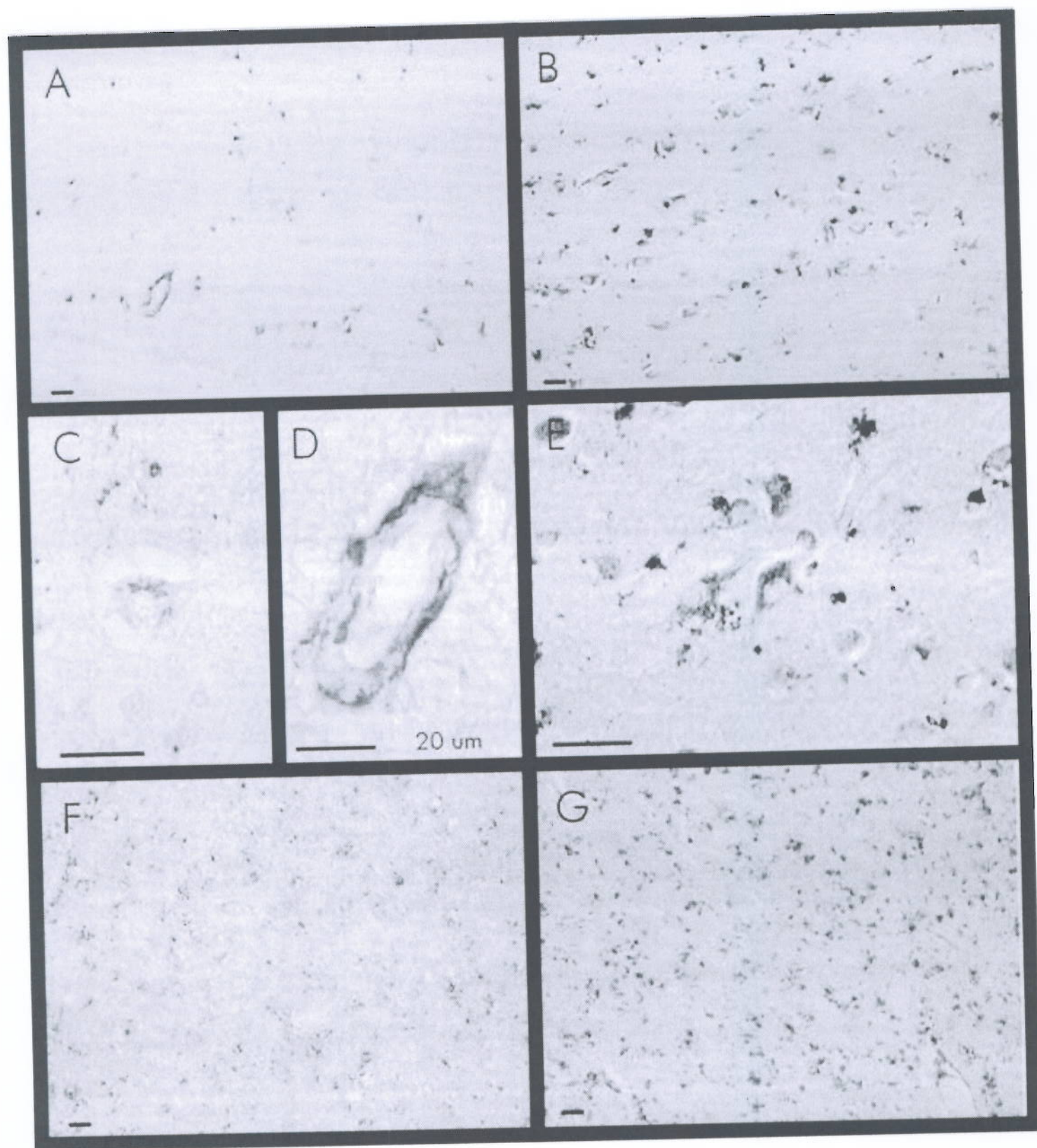


Figure 5

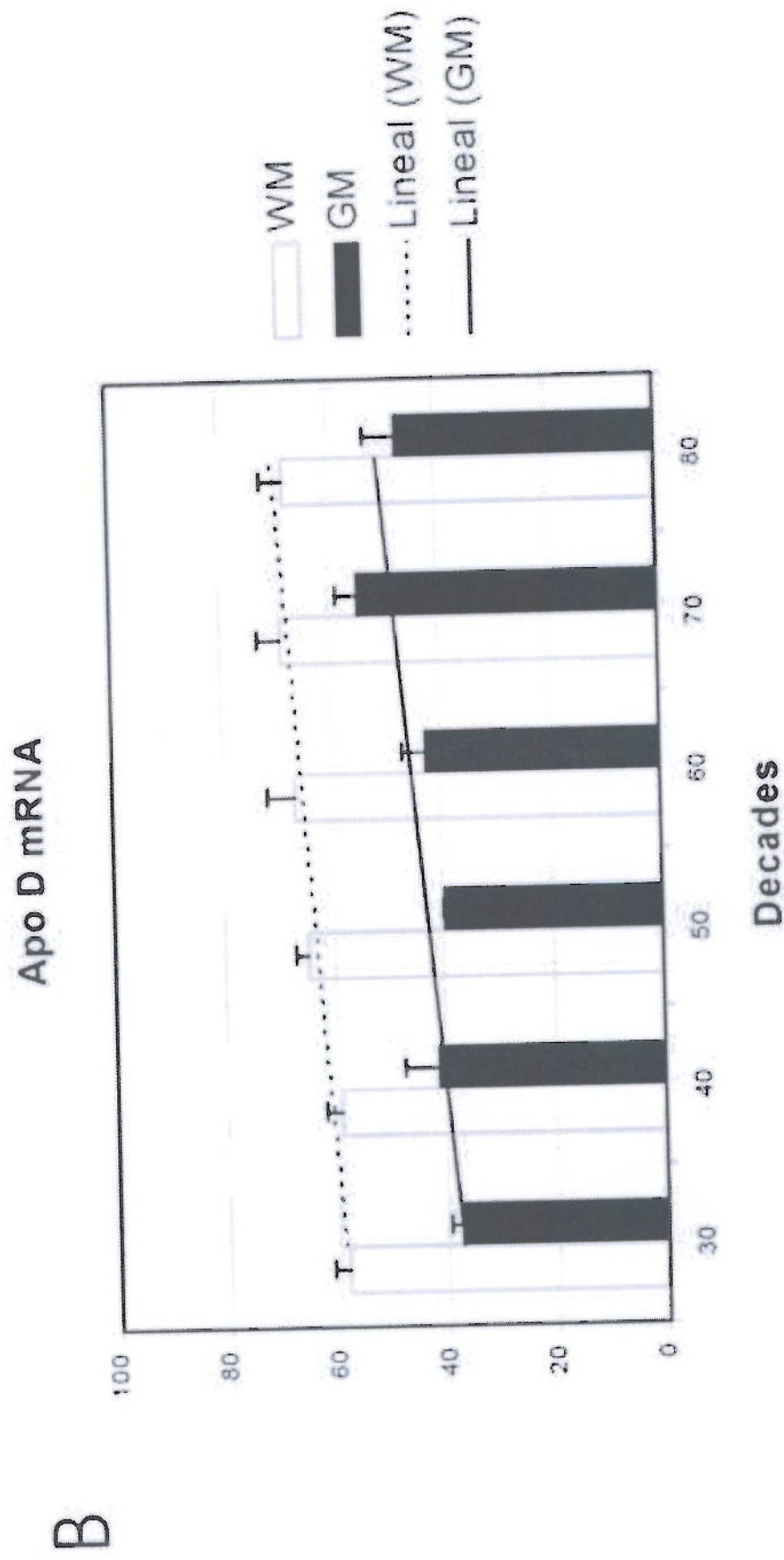


Figure 6

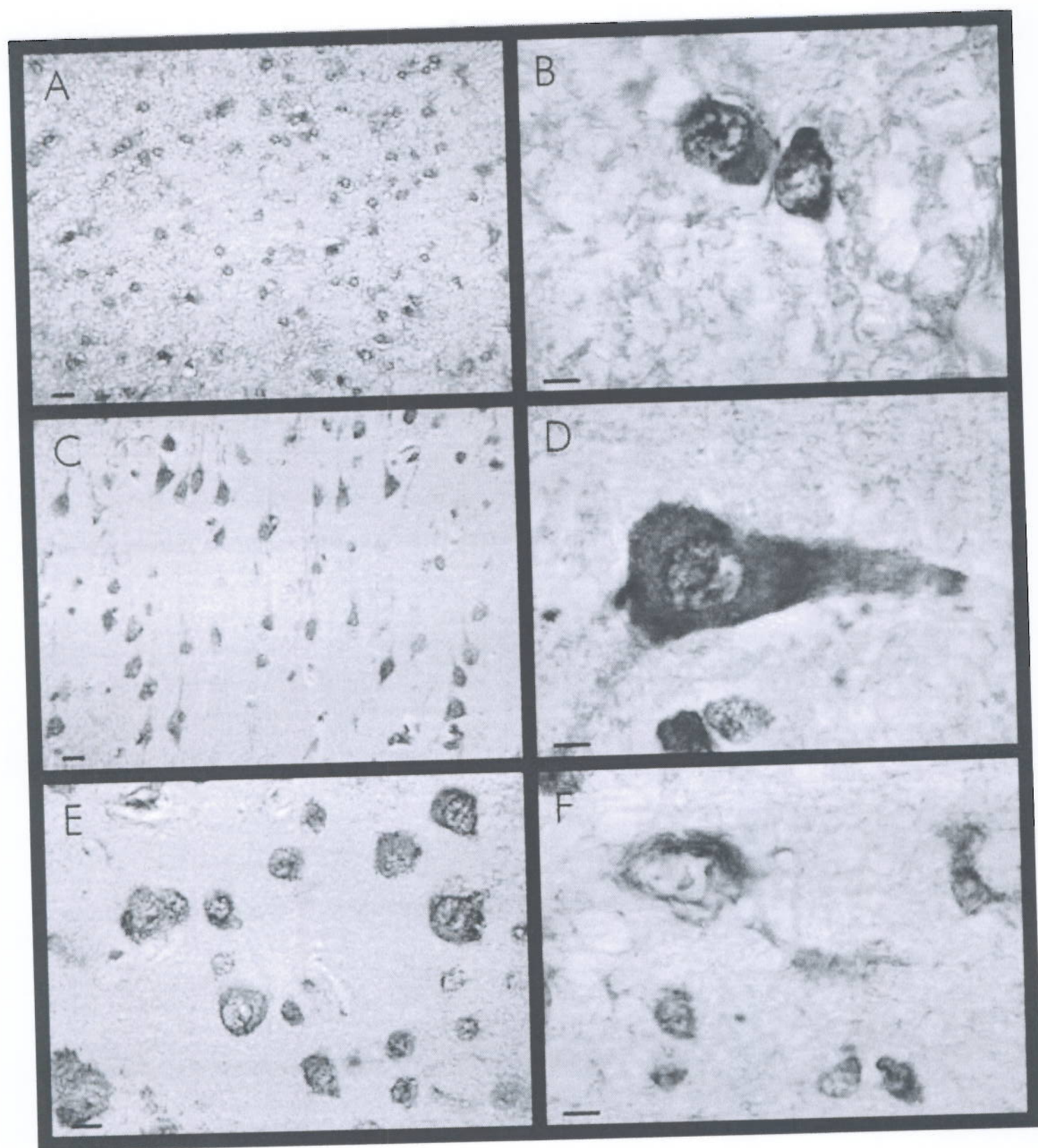


Figure 7

